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In conformance with Applicants' duty of disclosure under 37 C.F.R. § 1.56 (a) and § 1.97(b), the references listed on the attached form PTO-1449 are hereby brought to the Examiner's attention. In compliance with 37 C.F.R. § 1.98, a copy of the references are submitted herewith.

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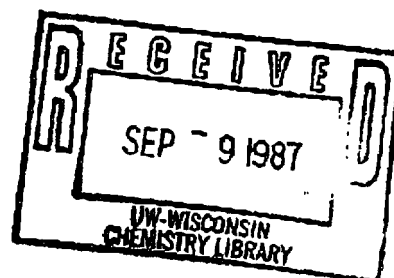
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No 20

Increased expression of the putative growth factor receptor p185^{HER2} causes transformation and tumorigenesis of NIH 3T3 cells

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ABSTRACT The *HER2* gene encodes a cell-surface glycoprotein with extensive homology to the epidermal growth factor receptor. Recently it was found to be amplified in about 30% of primary human breast malignancies. In experiments designed to assess the role of the *HER2* gene in oncogenesis, we found that overexpression of unaltered *HER2* coding sequences in NIH 3T3 cells resulted in cellular transformation and tumorigenesis.

The *HER2* gene encodes a transmembrane glycoprotein with extensive structural homology to the human epidermal growth factor (EGF) receptor and the chicken oncogene *v-erbB* (1-3). Chromosomal mapping and sequence comparison strongly suggest that the *HER2* gene product and the ethylnitrosourea-activated, rat neuroblastoma oncogene *neu* represent species variants of the same polypeptide (4). The *neu* oncogene encodes a 185-kDa cell-surface glycoprotein that possesses intrinsic tyrosine-specific kinase activity that is likely to be activated by an as yet unidentified ligand (5, 6). Comparison of the transforming *neu* oncogene sequence with its normal rat protooncogene counterpart suggested that a point mutation in the transmembrane domain resulting in substitution of a valine residue by glutamate unmasked the transforming potential of this putative growth factor receptor (7). Analogously, structural alterations have converted normal genes coding for the receptors for macrophage colony-stimulating factor type 1 and EGF into *v-fms* (8) and *v-erbB* (9) oncogenes, respectively.

Southern analysis of primary human tumors and established tumor-derived cell lines revealed amplification and in some cases rearrangement of the EGF receptor gene. Amplification was particularly apparent in squamous carcinomas (10, 11) and glioblastomas (12). The *HER2* gene was also found to be amplified in a human salivary gland adenocarcinoma (3), a mammary gland carcinoma (2), and a gastric cancer cell line (13). Recently, Slamon *et al.* (14) demonstrated that about 30% of primary human breast carcinoma tumors contained an amplified *HER2* gene. Although a few sequence rearrangements were detected, in most tumors there were no obvious differences between amplified and normal *HER2* genes. Furthermore, amplification of the *HER2* gene correlated significantly with the prognosis of the disease and the probability of relapse.

To investigate the significance of the correlation between overexpression and cellular transformation as it has been observed for protooncogenes *c-mos* (15) and *c-Ha-ras* (16), we employed a *HER2* expression vector and a selection scheme that permitted sequence amplification after transfection of mouse NIH 3T3 cells. We report here that amplification of the unaltered *HER2* gene in NIH 3T3 cells leads to overexpression of p185^{HER2} as well as cellular transformation and tumor formation in athymic mice. These findings, in

combination with the results of Slamon *et al.* (14), suggest that mere amplification of the *HER2* gene and resulting overexpression of its product may play a crucial role in the genesis and development of some types of human cancer.

MATERIALS AND METHODS

Expression Plasmids. The mammalian expression vector CVN (17) contained expression units for mouse dihydrofolate reductase (DHFR) cDNA (18) and the bacterial neomycin phosphotransferase (*neo*) gene (19), both under simian virus 40 early promoter control. Transcription of a 4.4-kilobase-pair *Sal* I-*Dra* I *HER2* fragment containing the full-length *HER2* coding region (1) was driven by the Rous sarcoma virus (RSV) long terminal repeat promoter (LTR). The poly(A) site was provided by the 3' untranslated sequence of the hepatitis B virus surface antigen gene (20). The control CVN plasmid was identical but lacked cDNA sequences downstream from the RSV LTR.

Cell Culture. NIH 3T3 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% HyClone (Logan, Utah) calf serum in a humidified incubator under 5% CO₂ in air atmosphere.

Transfections and Amplification. Plasmid DNA was introduced into mammalian cells by the calcium phosphate coprecipitation method (21). Half-confluent plates of cells (60 mm) were exposed to 5 µg of plasmid DNA in 1 ml of precipitate for 6-8 hr. After a 20% (vol/vol) glycerol shock (22), the cells were fed with nonselective medium. Two days later, they were passaged into selective medium containing Geneticin (G418) at 400 µg/ml.

Clones were picked using glass cloning cylinders with petroleum jelly for the bottom seal. Colonies arising from transfected cells selected for growth in G418 were picked, expanded, and subcultured into medium containing 7% dialyzed fetal bovine serum in place of 10% calf serum and the appropriate concentration of methotrexate for plasmid amplification (23). The dialysis step removes trace amounts of purines and pyrimidines present in serum that decrease the efficiency of the methotrexate selection. To apply selective pressure, stepwise increasing concentrations of methotrexate were used with a final concentration of 400 nM. To avoid enriching for spontaneously transformed cells, cells were kept subconfluent. An additional control was to amplify the CVN *neo*-DHFR vector without the *HER2* cDNA insert in the NIH 3T3 recipient cell line.

Immunoprecipitations and Labeling. The G-H2CT17 antibody recognizing the C-terminal 17 amino acids of *HER2* was prepared in rabbits using a synthetic peptide conjugated with soybean trypsin inhibitor.

Cells were harvested by trypsinization and counted by Coulter Counter, and 1.5×10^6 cells were plated per 60-mm culture dish. After 36 hr, the cells were lysed at 4°C with 0.4 ml of HNEG buffer per plate (50 mM Hepes, pH 7.5/150 mM NaCl/1 mM EGTA/10% glycerol) containing 1.0% Triton X-100 detergent and 1 mM phenylmethylsulfonyl fluoride. After 10 min, 0.8 ml of lysis dilution buffer (HNEG buffer with 1% bovine serum albumin and 0.1% Triton X-100) was added to each plate and the extracts were pelleted at $12,000 \times g$ for 5 min.

HER2 antibody was added to the cell extracts, which were then incubated at 4°C for 2 hr; this was followed by incubation with protein A-Sepharose beads for 20 min and three washes with 1 ml of HNEG buffer with 0.1% Triton X-100. Autophosphorylation reactions were carried out at 4°C in 50 μ l of HNEG wash buffer with 5 mM $MnCl_2$ and 3 μ Ci of [γ - ^{32}P]ATP (Amersham, 5000 Ci/mmol; 1 Ci = 37 GBq) for 20 min. Proteins were separated on 7.5% NaDodSO₄/polyacrylamide gels and analyzed by autoradiography.

Transformation Assays. The efficiency of colony formation in soft agar (24) was determined by plating 25,000 cells in 3 ml of 0.2% agar (Difco, "purified") over 4 ml of 0.4% agar in a 60-mm dish. After 2–4 weeks, colonies of about 100 cells or more were counted.

The plating efficiency of cell lines (25) in 1% calf serum was determined by plating equal numbers of cells into 100-mm plates with either 10% or 1% calf serum. After 2–3 weeks, the plates were stained with crystal violet and colonies were counted.

Mouse Tumorigenicity Assays. Athymic (*nu/nu*) mice were obtained from Charles River Breeding Laboratories. Control NIH 3T3 and NIH 3T3/CVN cells and experimental HER2-2₄₀₀ cells were harvested by trypsinization and counted with a Coulter Counter. They were then collected by low-speed centrifugation and resuspended in ice-cold phosphate-buffered saline to either 2.5×10^6 , 5.0×10^6 , or 1.0×10^7 cells per ml. Animals were injected subcutaneously with 0.1-ml volume of the cell suspensions. Tumor occurrence and size were monitored twice weekly.

RESULTS

For expression of *HER2* sequences in NIH 3T3 cells, a cDNA coding for the entire 1255-amino acid polypeptide (1) was placed under transcriptional control of the RSV LTR. Transcriptional termination signals and a poly(A) site were provided by 3' sequences of the hepatitis virus surface antigen gene (20). In addition, the expression vector contained the *neo* resistance (*neo*^R) gene, which confers cellular resistance to the aminoglycoside antibiotic G418 (18) and therefore allows selection of primary transfectants, as well as the *DHFR* gene for methotrexate resistance, which was used to amplify transfected DNA sequences under selective pressure. Both drug resistance genes were under simian virus 40 early promoter transcriptional control. Bacterial plasmid sequences, including an origin of replication and the gene for ampicillin resistance, allowed replication of the entire expression plasmid in *Escherichia coli*.

The transforming activity of *HER2* sequences was initially tested using a conventional NIH 3T3 cell focus-formation assay. Under conditions that resulted in about 10^4 foci per μ g of a *v-fms* viral construct, we were unable to detect any *HER2*-transforming activity. Because of the recently reported finding that about 30% of mammary carcinomas contain amplified *HER2* gene sequences without apparent sequence rearrangements (14), we investigated whether amplification of an unaltered *HER2* gene could transform mouse fibroblasts. NIH 3T3 cells were transfected with the pCVN/*HER2* construct. An identical plasmid missing the *HER2* expression module was used as a control. Four independent primary

Table 1. Assay for growth in soft agar and 1% calf serum of *HER2* primary and amplified cell lines

Cell line	<i>HER2</i> gene copies per haploid genome, no.	Soft agar colonies, no.	Plating efficiency in 1% calf serum, %
NIH 3T3/CVN	0	0	0.27
NIH 3T3/CVN ₄₀₀	0	0	0
HER2-1	3	1	3
HER2-1 ₄₀₀	60	424	38
HER2-3	2	0	1.4
HER2-3 ₄₀₀	55	836	11.7
HER2-4	4	0	0.2
HER2-4 ₄₀₀	90	376	49
HER2-B3	1	0	0.6
HER2-B3 ₄₀₀	131	373	50.2

Two control lines were used. The first one was a NIH 3T3 line transfected with a plasmid containing only the *neo* and *DHFR* genes. The second control line contained the *neo*-*DHFR* plasmid and was amplified to resistance to 400 nM methotrexate. *HER2* gene copy numbers were determined using a human DNA standard and densitometer scanning of Southern hybridization autoradiograms. Equal cell numbers (25,000) were plated in soft agar and colonies were counted after 2–4 weeks. The plating efficiency in 1% calf serum is relative to the number of colonies arising when an equal aliquot was simultaneously plated in medium containing 10% calf serum.

G418-resistant clones (HER2-1, HER2-3, HER2-4, HER2-B3) were isolated. Cell lines containing amplified *HER2* coding sequences were generated from these parental clones by culturing the cells in gradually increasing concentrations of methotrexate up to 400 nM (HER2-1₄₀₀, HER2-3₄₀₀, HER2-4₄₀₀, HER2-B3₄₀₀). Southern hybridization analysis of parental and amplified cell lines demonstrated that the *HER2* cDNA copy number increased from 1–4 to 55–131 per haploid genome (Table 1).

To test whether gene amplification resulted in overexpression of the *HER2* gene product, cell lysates were immunoprecipitated with an antibody against the C-terminal 17 amino acids of the *HER2* sequence. As shown in Fig. 1, substantially increased levels of the p185^{HER2} gene product were found in amplified cell lines relative to their parental G418^R transfectants. The parental cells had a normal morphology that was indistinguishable from NIH 3T3 cells. However, amplified cells had the typical refractile, spindle-

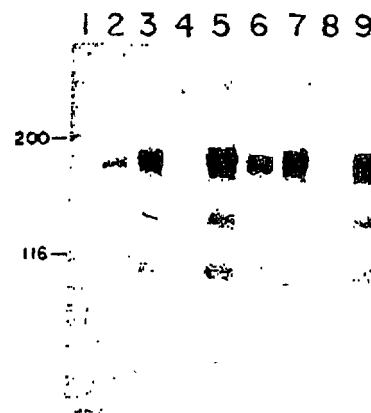


FIG. 1. Quantitation of p185^{HER2} in four primary, unamplified cell lines and lines derived from them by amplification to resistance to 400 nM methotrexate. Lane 1, *neo*-*DHFR* control; lanes 2 and 3, HER2-1 parent and amplified lines; lanes 4 and 5, HER2-3 parent and amplified lines; lanes 6 and 7, HER2-4 parent and amplified lines; lanes 8 and 9, HER2-B3 parent and amplified lines. Positions of the size markers myosin and β -galactosidase are indicated in kDa.

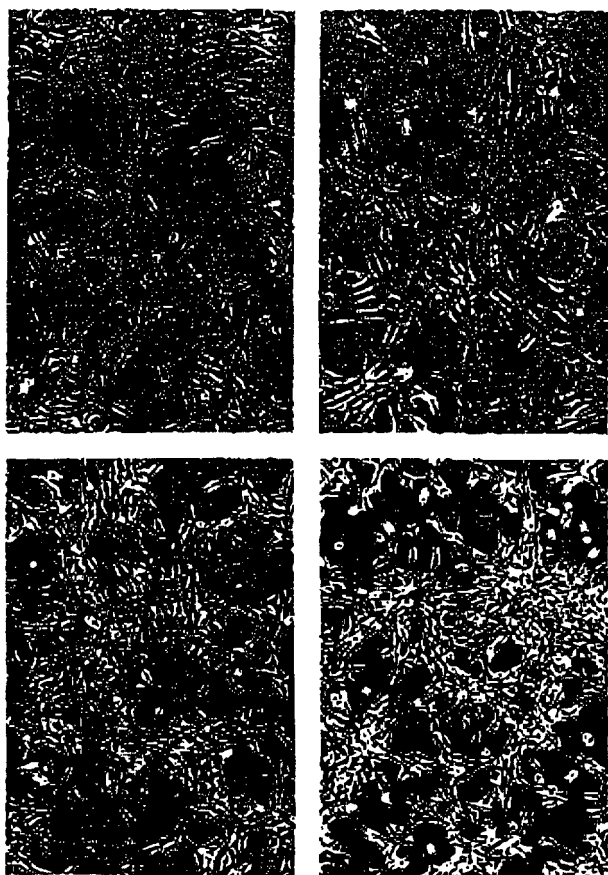


FIG. 2. Morphology of NIH 3T3 cells transfected with *HER2* expression construct. (1) NIH 3T3 cells transfected with the control neo-DHFR plasmid. (2) NIH 3T3 cells with the neo-DHFR plasmid amplified to resistance to 400 mM methotrexate. (3) Primary G418^R cell line HER2-1, an unamplified clone expressing low levels of p185. (4) The same clone amplified to 400 mM methotrexate resistance. ($\times 50$.)

shaped appearance of transformed cells and grew in irregular, piled-up clumps (Fig. 2).

NIH 3T3 cell lines with an amplified *HER2* gene and high levels of *HER2* gene expression also displayed other characteristics associated with a transformed phenotype. As shown in Table 1 and Fig. 3, these cells all formed colonies in soft agar and were able to grow at low density at low serum concentration. In contrast, primary transfectants did not grow under these conditions. The primary transfectants did, however, grow to a higher saturation density than the parental NIH 3T3 cells.

The correspondence between a transformed phenotype and *HER2* gene amplification and overexpression was independently confirmed by directly selecting for transformed cells and then analyzing the resulting clones. For this purpose the parental cell line HER2-3, which contains about two copies of the *HER2* expression construct, was cultured in medium containing a low concentration of fetal calf serum (0.5%); control cells containing the expression vector without *HER2* coding sequences (pCVN) were cultured in parallel. After 5 weeks, a few colonies appeared in the control culture and roughly 10-fold more colonies appeared in dishes containing the HER2-3 cell line. These colonies appeared to be morphologically transformed and were subsequently analyzed for *HER2* overexpression. As shown in Fig. 4, three individual clones as well as a pool of the remaining colonies had elevated levels of p185^{HER2} compared with the original parental G418-resistant HER2-3 cell line. In addition, there

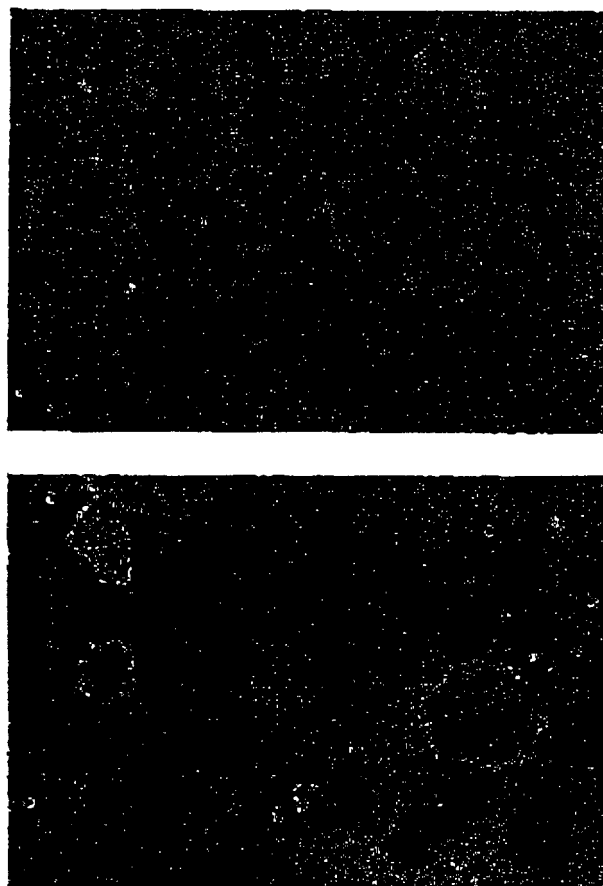


FIG. 3. Anchorage-independent growth of *HER2*-transformed cells in soft agar. Cells were plated in 0.2% soft agar over a 0.4% agar lower layer. After 3 weeks the plates were photographed at 40 \times magnification using a Nikon microscope with phase-contrast optics. (Upper) Control untransformed NIH 3T3 cells. (Lower) Anchorage-independent growth of the cell line HER2-3₄₀₀ containing the *HER2* expression plasmid and amplified to resistance to 400 mM methotrexate. ($\times 25$.)

was a 26-fold increase in the number of cells plating in 100 nM methotrexate in the selected cells compared with the parental cells, implying that the unselected but linked *DHFR* gene had also been coamplified.

The tumorigenicity of cell lines with a high *HER2* cDNA copy number was tested in nude mice by subcutaneous

1 2 3 4 5 6

—185

FIG. 4. Quantitation of p185 in the primary line HER2-3 and cell lines selected for growth in 0.5% calf serum. *HER2*-encoded protein was immunoprecipitated and labeled. Lanes 1–3, three colonies picked and expanded from plates after selection for growth in 0.5% serum; lane 4, the starting cell line, HER2-3; lane 5, a pool of 0.5% serum-selected colonies of HER2-3; lane 6, a clone derived from a G418^R control line selected for growth in 0.5% calf serum. The position expected for a protein of apparent molecular mass of 185 kDa is indicated.

Table 2. Tumorigenicity testing of cell lines

Cell line	Cells injected, no.	Mice with tumors/ mice injected
NIH 3T3	0.25×10^6	0/6
	0.5×10^6	0/6
	1.0×10^6	0/6
NIH 3T3/CVN	0.25×10^6	0/6
	0.5×10^6	0/6
	1.0×10^6	0/6
HER2-3 ₄₀₀	0.25×10^6	5/5
	0.5×10^6	6/6
	1.0×10^6	5/5

The cell line HER2-3 and two control lines, NIH 3T3 cells and a NIH 3T3 line containing the control plasmid, were injected subcutaneously at three different dosages into nude mice. The time after injection before tumors became visible was dose related: 22 days (average) for 1×10^6 cells, 28 days (average) for 0.5×10^6 cells, and 34 days (average) for 0.25×10^6 cells.

injection of three different cell numbers per animal. As shown in Table 2, the overexpressing cell line HER2-3₄₀₀ was strongly tumorigenic at all dosages tested, whereas the control cell lines, NIH 3T3 cells and NIH 3T3 cells transfected with CVN vector without the HER2 insert, were both negative under the same conditions. Necropsy of three mice with well-established tumors failed to identify any metastasis. Cell lines reestablished from three excised tumors still expressed the G418^R phenotype, were resistant to methotrexate, and expressed high levels of p185^{HER2} (not shown).

DISCUSSION

Amplification of the *HER2* gene has been reported in a few primary human tumors (2, 3) and tumor-derived cell lines (13, 26). Recently, Slamon *et al.* (14) found that the *HER2* gene is amplified in 30% of primary breast tumors, a common human malignancy that affects about 7% of all American females. Notably, only 3/189 tumors surveyed showed any gross rearrangement of the *HER2* gene. To assess the role of the *HER2* gene in the neoplastic process, we characterized the transforming potential of the *HER2* gene in an *in vitro* cell culture system.

Expression of the full-length cDNA in NIH 3T3 cells did not lead to transformation as determined by a standard focus-forming transfection assay. However, *HER2* overexpression caused by gene amplification transformed these cells. Colonies that survived methotrexate selection were morphologically transformed and exhibited loss of contact inhibition. Such cells also grew in soft agar and would grow in 1% calf serum. Furthermore, cells transformed by *HER2* were tumorigenic in athymic mice.

Selection of cells transfected with the *HER2* cDNA for growth in low serum provided independent evidence that high-level expression of an unaltered *HER2* gene product caused cellular transformation. Since DNA introduced into mammalian cells by transfection is more labile than genomic DNA (23, 27), we reasoned that selection for a property demonstrated by pharmacologically amplified *HER2* lines—namely, growth in low serum—might lead to amplification or other changes resulting in overexpression of p185^{HER2}. Clones derived from an unamplified *HER2* line by this selection procedure appeared morphologically transformed and exhibited elevated levels of p185^{HER2}.

Taken together, the characteristic morphological changes, results of *in vitro* and *in vivo* transformation and tumorigenicity assays, and the elevated levels of p185^{HER2} in cells selected for a transformed phenotype imply that high-level expression of *HER2* results in transformation of NIH 3T3

cells. Another member of the tyrosine kinase gene family was recently found to be amplified 4- to 8-fold in spontaneously arising foci of NIH 3T3 cells (28). Amplification of the *met* gene appears to be a frequent event and, similar to *HER2* amplification in mammary carcinomas, is rarely accompanied by gross rearrangements. These findings and the discovery of amplified EGF receptor genes in primary human tumors (10–12) suggest that overexpression of other growth factor receptor genes will also lead to transformation and tumorigenesis. Whether susceptibility to spontaneous amplification is caused by characteristics of the genetic loci for *HER2*, *met* and EGF receptor, or cell-specific selective advantages caused by receptor overexpression remains to be investigated.

It is not yet clear whether transformation of cells overexpressing a growth factor receptor gene is dependent on paracrine or autocrine stimulation by the appropriate ligand. For *HER2*, this question cannot yet be addressed since its ligand has not been identified. In the case of the EGF receptor, however, we were able to demonstrate that primary human tumors and tumor-derived cell lines frequently express mRNAs for both the receptor and transforming growth factor type α ligand (29). The finding that mere overexpression of an intact growth factor receptor may subvert normal cellular growth control mechanisms and lead to tumorigenic growth provides new potential for diagnostic approaches and therapeutic strategies for treatment of human malignancies.

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